Technical Overview

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Hydrophilic Interaction Chromatography Method Development and Troubleshooting

Introduction

Hydrophilic interaction chromatography (HILIC) is a rapidly growing technique within the field of high-performance liquid chromatography (HPLC). Even the most polar compounds can be separated with HILIC using the same system and solvents as reversed-phase HPLC.

HILIC eliminates much of the cost and labor for polar analysis, compared to older techniques such as derivatization, ion exchange, ion pairing, and normal-phase HPLC. However, method development and troubleshooting can pose a challenge even to experienced chromatographers, and only recently have HILIC columns been able to offer performance and reproducibility comparable to better-established reversed-phase chemistries.

This Technical Overview reviews the basics of HILIC and method development, and concludes with troubleshooting.

Understanding HILIC and polar analysis

Columns operating in HILIC mode retain moderate to highly polar compounds. This range of compounds overlaps slightly with C18 in reversed-phase HPLC, which retains moderate to nonpolar compounds.

HILIC stationary phases are highly polar and hydrophilic, which causes them to absorb water from the mobile phase to form a thin layer of water on the surface. Approximately 3 % water is required to form this film, depending on the exact stationary phase. Increasing the water content of the mobile phase reduces the partitioning effect, which disappears completely when the mobile phase contains approximately 50 % water. At this point, compounds are no longer retained by the HILIC mechanism.

More polar mobile phases are stronger solvents, the opposite of reversed-phase HPLC. Water is the strongest mobile phase in HILIC, followed by methanol. Figure 1 shows that solvent strength increases. Buffer salts also increase a solvent's strength, but not as significantly.

Figure 1. Solvent strengths.

Note that unlike reversed-phase HPLC, pure methanol (MeOH) is too strong a solvent for most HILIC separations. Acetonitrile (ACN) is the recommended weak solvent, while alcohols can be added to increase solubility of buffers or to alter selectivity slightly.

Techniques for polar compounds – HILIC compared to other methods

Various techniques exist for analysis of polar compounds. These different approaches each come with advantages and disadvantages, as listed in Table 1.

By comparison, HILIC is a powerful and flexible technique, see Table 2.

Table 1. Advantages and disadvantages of historical techniques.

Technique	Advantages	Disadvantages
Ion pairing	Fast analysis Uses standard HPLC system and reversed-phase columns	Often contaminates system Can cause ion suppression Restricts operation to only positive or negative mode MS
Ion chromatography	Simple mechanism, strong retention, and predictable separation Unique detection modes	Slower than modern HPLC/UHPLC, expensive systems, and consumables Cannot resolve cations and anions simultaneously Challenging to make MS-compatible
Ion exchange	Simple mechanism, strong retention, and predictable separation Media is cheap and available	Slower than HPLC Cannot resolve cations and anions simultaneously Challenging to make MS-compatible
Normal phase	Fast analysis Uses standard HPLC system and common columns	Safety and compatibility of organic solvents Smaller selection of stationary phases Sample solubility issues
Derivatization	Fast analysis Tailored selectivity Add a chromophore or fluorophore	Lengthy sample preparation Repeatability issues Complex MS spectra Toxic derivatization agents

Table 2. Advantages and disadvantages of hydrophilic interaction chromatography.

Technique	Advantages	Disadvantages
HILIC	Fast analysis Uses the same system and solvents as reversed-phase HPLC Separates cations, anions, and polar neutrals in a single run Equal or superior MS performance to reversed-phase HPLC	Sample solubility in high organic solvents 100 % Methanol not suitable as organic phase Hardware inertness requirements

Getting started with HILIC

Choosing a column is the first step in developing a HILIC method. In contrast to nonpolar reversed-phase stationary phases, HILIC columns use a polar stationary phase. The oldest and most widely available HILIC phase is bare silica. However, the variability and acidity of the bare silica surface also makes it challenging to reliably analyze many common classes of polar compounds. Various bonded HILIC phases have been developed to offer alternative selectivity and improved reliability. Newer HILIC phases are now able to offer resolution, peak shape, and reliability equivalent to reversed-phase chemistries.

In addition to an appropriate column, HILIC also requires some changes to the running parameters that would be used for a reversed-phase separation. These changes relate to the inverted relationship between polarity and retention: polar compounds are retained, nonpolar compounds are unretained, polar solvents are strong, nonpolar solvents are weak.

Starting parameters

Stationary phase:

- Different stationary phases have different selectivity.
- Modern bonded phases offer superior peak shape and minimal secondary interactions from ion exchange.
- Three phases on Agilent InfinityLab Poroshell 120, 2.7 µm particles:
  - **InfinityLab Poroshell HILIC-Z –** Zwitterionic chemistry with a proprietary bonding technique that offers powerful separation, stability across wide a wide pH range, and excellent peak shape
  - **InfinityLab Poroshell HILIC-OH5 –** Fructan chemistry with alternative selectivity and excellent peak shape for a wide range of polar compounds
  - **InfinityLab Poroshell HILIC –** Traditional bare silica phase

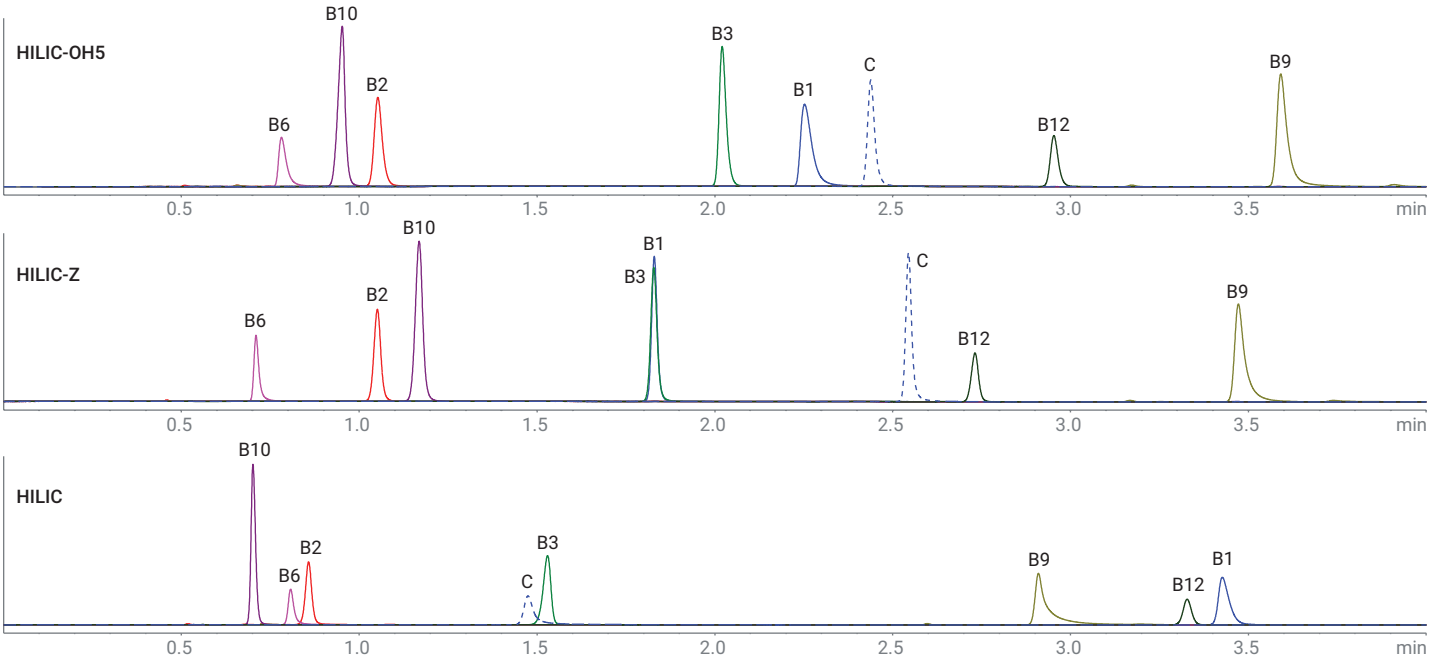

Figure 2. Separation of water-soluble vitamins. Columns: 2.1 × 100 mm, 2.7 µm, see figure for phases. Mobile phase A: 100 mM ammonium acetate + 0.5 % acetic acid (approximately pH 4.6) in water. Mobile phase B: acetonitrile. Flow: 0.5 mL/min. Gradient: 87 %B for one minute, 87 to 50 %B in four minutes. Re-equilibration: three minutes. Injection: 1 µL of individual vitamin standards (0.1–0.4 mg/mL each). Column temperature: 40 °C. Detection: UV, 260 nm, 80 Hz.

Mobile phase composition:

- Water is the strong solvent. Reduced water content increases retention.
- Less polar compounds require high organic to be retained.
- Increasing buffer concentration decreases retention and improves peak shape, but can affect detector response.

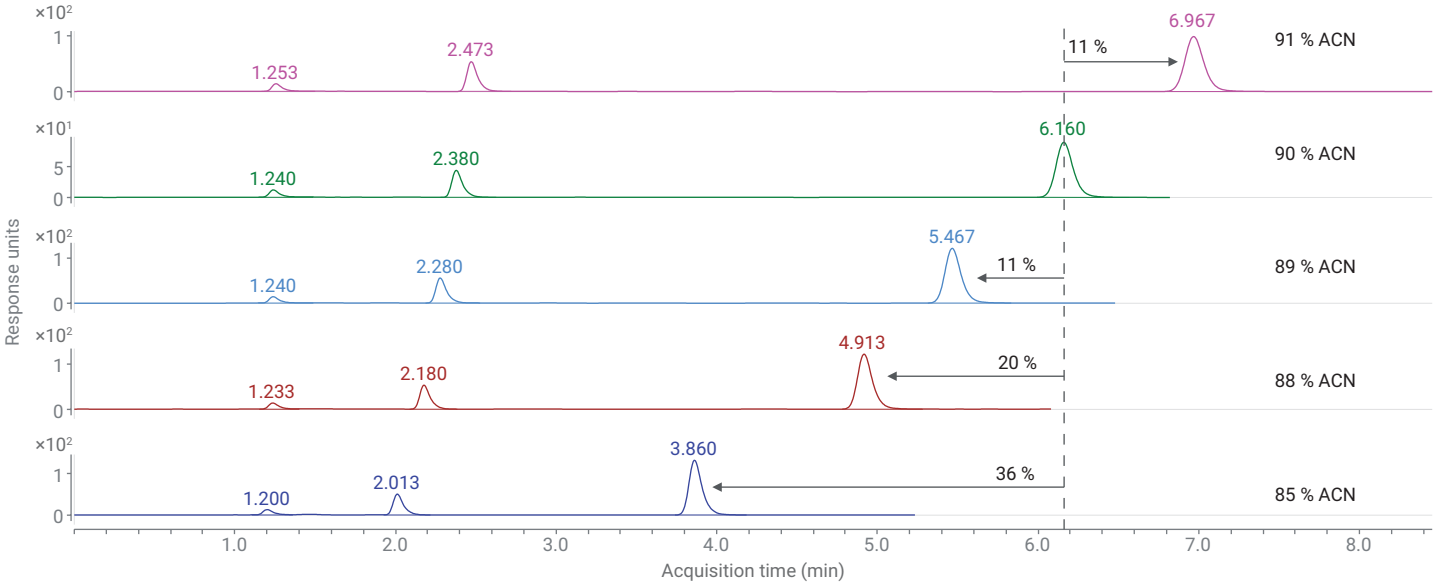

Figure 3. HILIC analyses can be sensitive to small changes in mobile phase composition. Column: InfinityLab Poroshell 120 HILIC-Z (PEEK lined) 2.1 × 150 mm, 2.7 µm. Mobile phase A: 100 mM pH 3 ammonium formate in water, pH 3. Mobile phase B: acetonitrile. Isocratic elution: see figure for %B. Flow: 0.25 mL/min. Column temperature: 30 °C. Injection: 1 µL of toluene, cytosine, uracil QC mixture. Detection: UV, 254 nm.

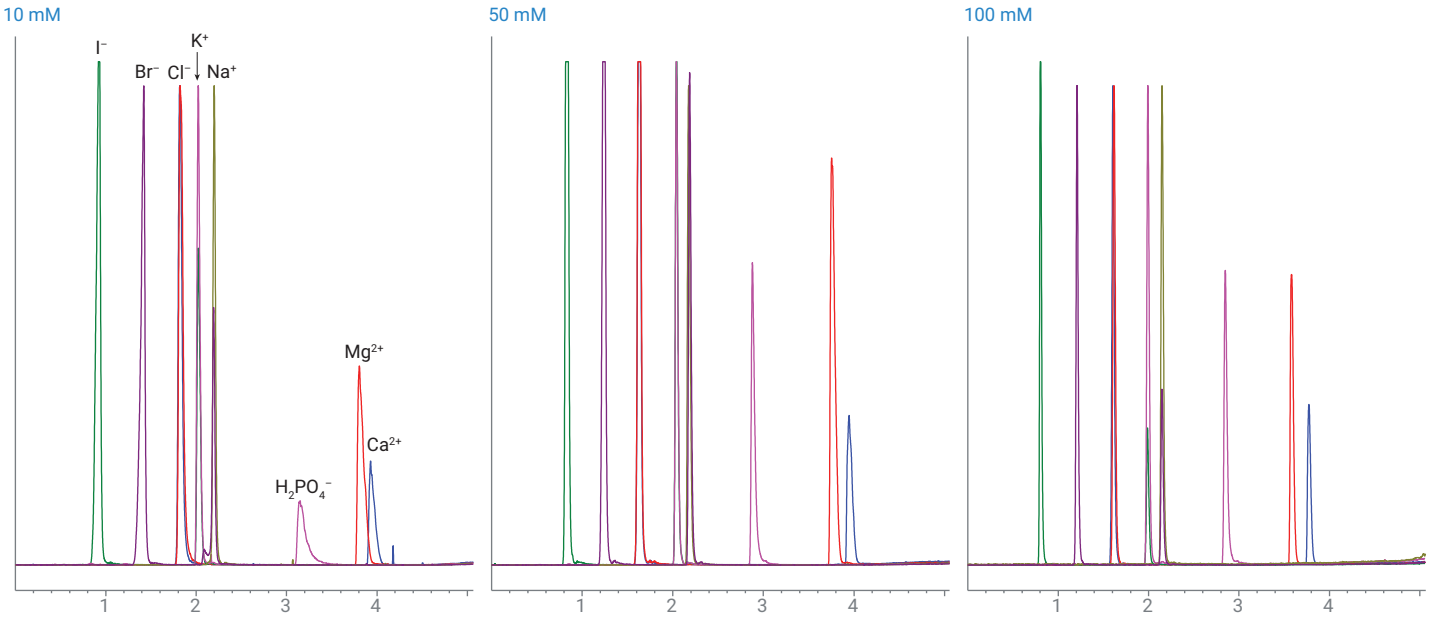

Figure 4. Separation of inorganic ions using an InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm column. Mobile phase A: 10, 50, or 100 mM pH 3 ammonium formate. Mobile phase B: Acetonitrile. Gradient: 80 to 20 %B in five minutes. Re-equilibration: three minutes. Flow: 0.4 mL/min. Column temperature: 30 °C. Injection: 2 µL injection of individual standards (0.3–0.5 mg/mL). Detection: ELSD, 40 °C, 3.5 psi, 30 Hz.

Mobile phase pH:

- Controls ionization of samples and particle surface charge (a secondary mechanism on silica, NH2, and older bonded HILIC phases)
- Compounds are more retained in their charged state, the opposite of reversed-phase
  - Acids should be run at high pH, bases at low pH

Temperature:

- Increasing temperature will decrease retention
- Increasing temperature will increase column efficiency
- Decreasing temperature can improve selectivity

Mobile phases

General mobile phases:

Low pH	Mobile phase A: 10–20 mM ammonium formate, pH 2.8 to 4.8 with formic acid Mobile phase B: 20 mM ammonium formate in 90:10 ACN:H_2O, pH 2.8 to 4.8 with formic acid
Moderate pH	Mobile phase A: 10–20 mM ammonium acetate, pH 3.8–5.8 with acetic acid Mobile phase B: 10 mM ammonium acetate in 90:10 ACN:H_2O, pH 4.8 to 7 with acetic acid
High pH	Mobile phase A: 0.3 % ammonium hydroxide in water Mobile phase B: 0.3 % ammonium hydroxide in ACN

Starting conditions:

Isocratic conditions	90 % ACN – Less polar analytes 80 % ACN – Polar analytes, mixtures 70 % ACN – Very polar analytes 50 % ACN – Column wash (no retention, recommended postanalysis when operating at >80 % ACN in isocratic mode)
Gradient conditions	90 % to 50 % ACN – Scouting gradient Isocratic holds or shallow gradients (1 to 3 % per min) recommended for critical pair separation

Notes:

- High-phosphate buffers are not recommended. Further details can be found in the Buffer Solubility section.
- At high organic concentrations, retention can be sensitive to small changes in organic concentration. Solvents must be measured accurately and mixers must be in good working order.
- MeOH and other alcohols can be mixed with acetonitrile in small quantities (<25 %) to change selectivity slightly.
- Increasing buffer concentrations general improves peak shape, but decreases sensitivity in LC/MS, and generates baseline noise in ELSD.
- Operating within the buffering range of the added salts is recommended, but not critical for analysis, especially at low sample loadings
  - Ammonium acetate: pH 2.8 to 4.8, 8.2 to 10.2
  - Ammonium formate: pH 3.8 to 5.8, 8.2 to 10.2

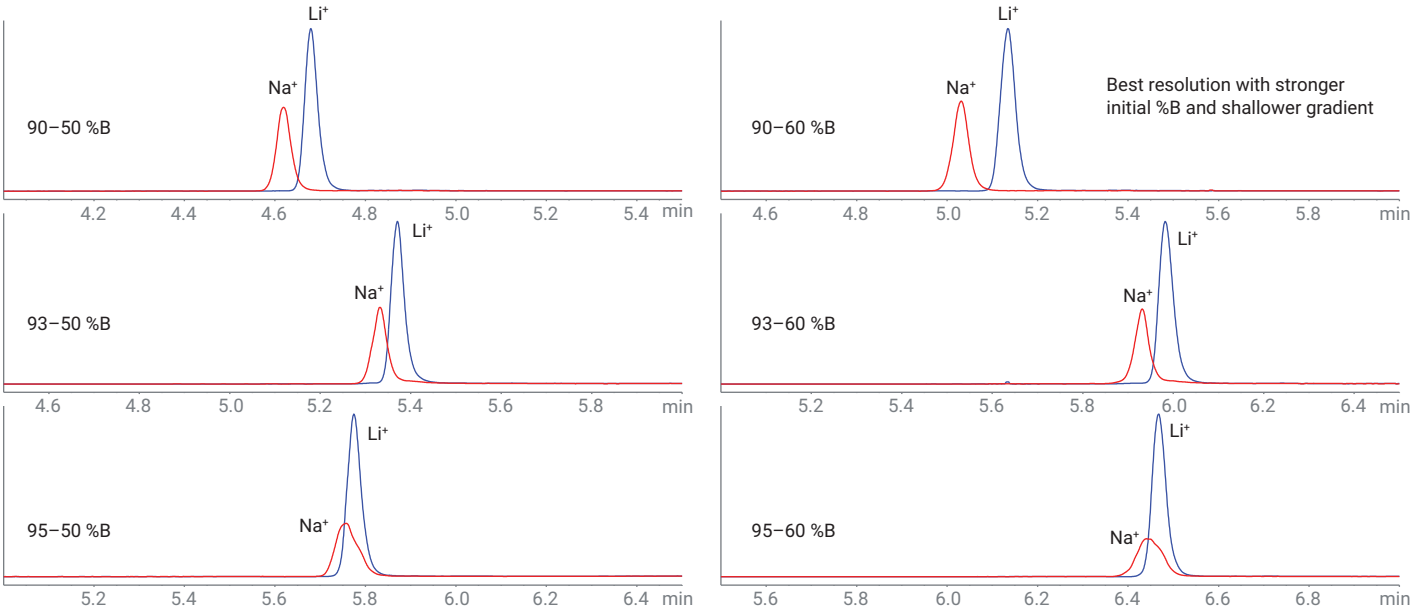

Figure 5. Resolving a critical pair (Na+ and Li+) using an InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm column. Mobile phase A: 100 mM ammonium formate, pH 3. Mobile phase B: acetonitrile. Gradient: see figure for %B in 10 minutes. Re-equilibration: three minutes. Flow: 0.4 mL/min. Column temperature: 30 °C. Injection: 2 µL of individual standards (0.3–0.5 mg/mL). Detection: ELSD, 40 °C, 3.5 psi, 30 Hz.

Equilibration

Column equilibration and re-equilibration are directly related to the water content of the mobile phase. Each run requires that the water layer be refreshed with high-aqueous mobile phase, after which the concentration of water in the mobile phase can be reduced to the starting conditions of the next run.

If the column has been exposed to at least 20 % water, either in the gradient or as a wash step, the column will quickly equilibrate when it is brought back to high-organic conditions. Most gradient analyses reach this concentration easily, so re-equilibration times are similar to reversed-phase analyses.

Analyses operating in isocratic conditions with high-organic conditions may require a brief wash postrun in high-aqueous mobile phase to remove strongly retained compounds, such as inorganic salts and other polar compounds.

Buffer solubility

Pure acetonitrile does not easily dissolve many common buffers, even ammonium acetate and formate. Mixing 90:10 of acetonitrile:water drastically improves buffer solubility. Some common reversed-phase buffer salts, such as phosphate, are highly insoluble across most of the mobile phase compositions used for HILIC.

Severe clogging and potential damage to the system can occur if a concentrated aqueous buffer is mixed with enough organic solvent to cause the buffer to precipitate. This can easily occur when using buffers containing sodium, potassium, phosphate, or borate ions.

One best practice to prevent this is to premix the high organic phase with the buffer in the organic solvent bottle, and observe for any cloudiness or crystallization. This should be done for buffers containing sodium, potassium, phosphate, or borate ions in any amount, and for buffers containing >10 mmol ammonium acetate or formate with >90 % acetonitrile.

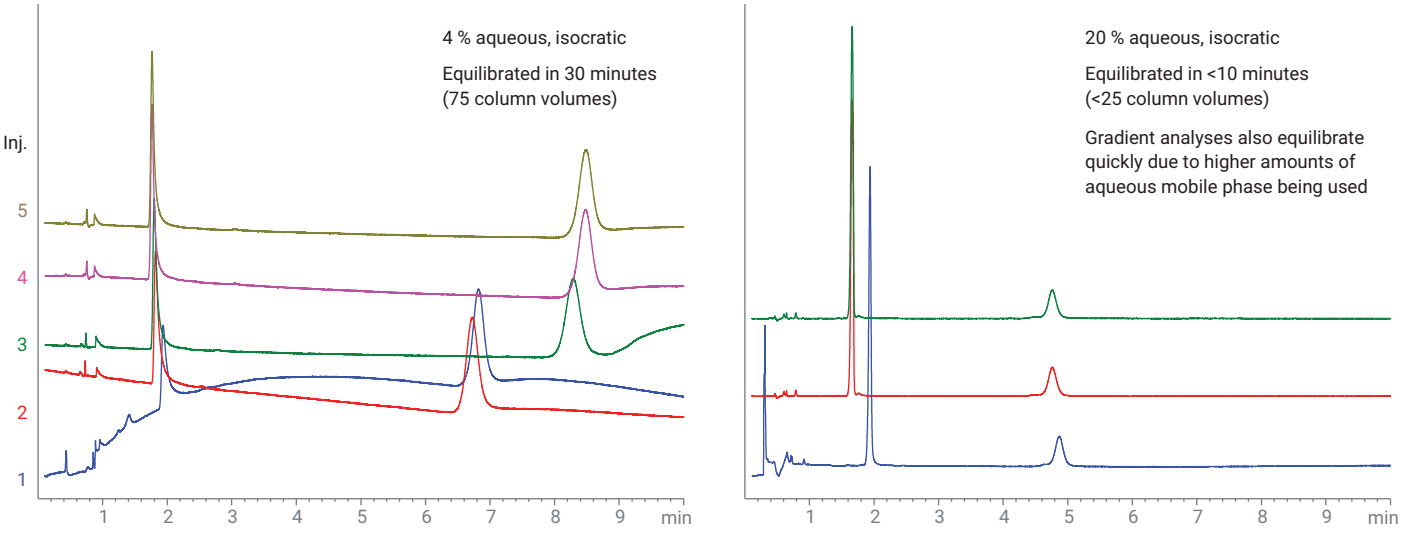

Figure 6. Separation of B vitamins using an InfinityLab Poroshell 120 HILIC-OH5, 2.1 × 100 mm, 2.7 µm column. Left: Column stored in 100 % acetonitrile prior to analysis. Mobile phase A: 100 mM ammonium formate, pH 3.0. Mobile phase B: acetonitrile. Isocratic conditions: 96 %B. Flow: 0.5 mL/min. Injection: 1 µL of B2+B6. Column temperature: 25 °C. Detection: 260 nm, 80 Hz. Right: Column stored in 100 % acetonitrile prior to analysis. Mobile phase A: 100 mM ammonium formate, pH 3.0, Mobile phase B: acetonitrile. Isocratic conditions: 80 %B. Flow: 0.5 mL/min. Injection: 1 µL of B9+B12. Column temperature: 25 °C. Detection: 260 nm, 80 Hz.

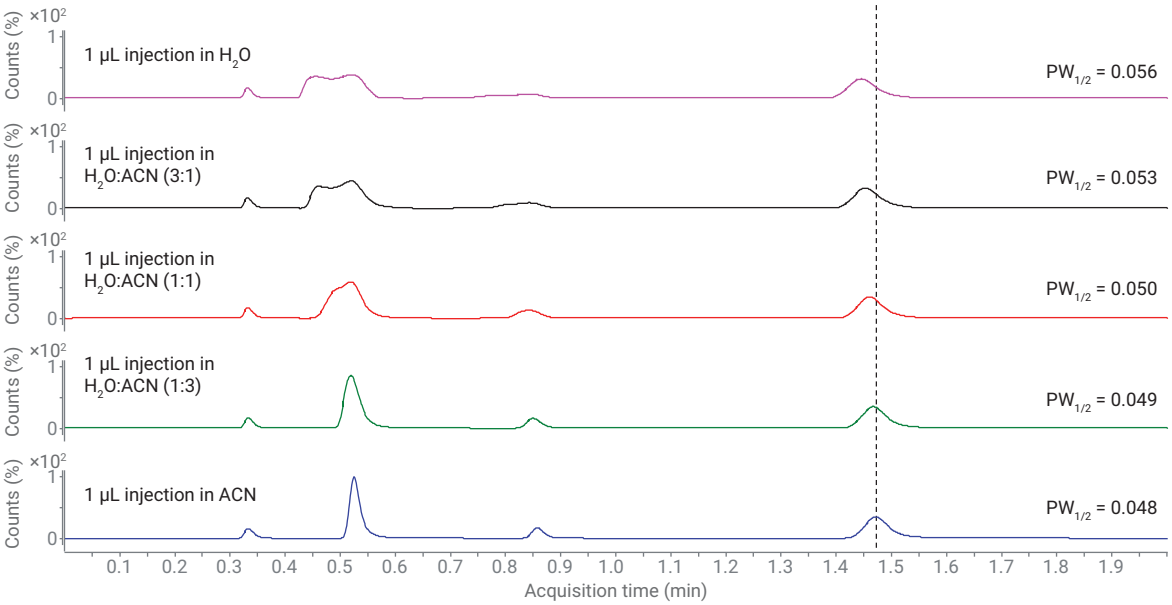

Figure 7. Separation of B vitamins on HILIC with isocratic elution. Agilent ZORBAX RRHD HILIC Plus 2.1 × 50 mm, 1.8 µm; Mobile phase: acetonitrile/100 mM Ammonium Formate pH 3.2 in water (9:1), isocratic elution, 0.4 mL/min, 1 µL injection of 5.7 µg/mL each of 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid ; 25 °C, MS Source: ESI+, 200 °C, 10 L/min, 30 psi, 4,000 V; SIM: 138, 123, 377, 124.

Sample solvent and injection volume

Because water is a highly strong solvent in HILIC mode, samples dissolved in 100 % aqueous solvents can be poorly retained on the column, and suffer from poor peak shape. This is analogous to injecting a sample dissolved in 100 % chloroform or hexane onto a reversed-phase column.

For compounds that are only soluble in water, injection volumes should be kept low enough to eliminate any variability.

The exact onset of peak splitting varies by by analysis. The following table gives the recommended maximum starting value for most methods:

Sample solvent	2.1 × 50 mm	3.0 × 50 mm	4.6 × 50 mm
100 % H_2O	≤1 µL	≤2 µL	≤3 µL
50:50 ACN:H_2O	≤2 µL	≤4 µL	≤6 µL
80:20 ACN:H_2O	≤5 µL	≤10 µL	≤15 µL

Longer columns can handle higher injection volumes; the following multipliers can be used for the values above:

Column length	50 mm	100 mm	150 mm
Injection volume	1x	1.5x	2x

Peaks broaden with increasing concentration, and when this begins to affect peak shape and resolution, the column is overloaded. More concentrated samples should always be injected at lower volumes, or be diluted.

Dilute solutions are always recommended for LC/MS, since interfering compounds can be isolated as narrow peaks that do not obscure the analytes of interest. This is powerful because HILIC retains inorganic ions, so sodium, potassium, and organic impurities can be separated. Higher loadings can cause these interference peaks to become broader, and potentially overlap and interfere with key compounds.

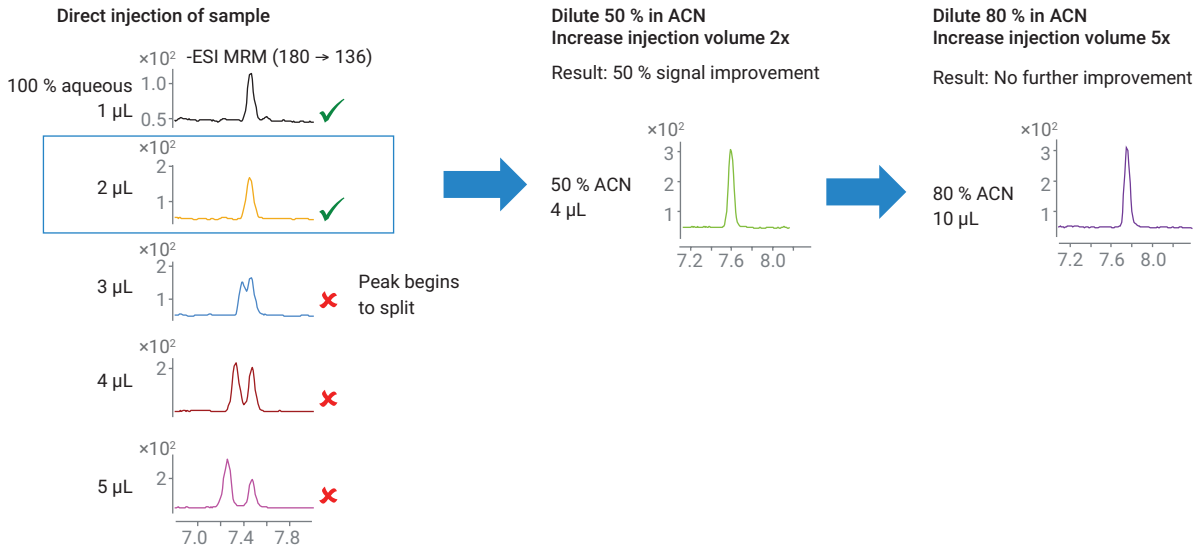

Figure 8. Sensitivity can be maintained or improved with acetonitrile dilution and increased injection volume. System: Agilent 6490 triple quadrupole LC/MS. Sample: glufosinate, 100 ppb. Column: InfinityLab Poroshell HILIC-Z, 2.1 × 100 mm. Mobile phase A: 10 mM ammonium acetate, pH 9. Mobile phase B: 100 mM ammonium acetate, pH 9 in 90 % acetonitrile (final concentration: 10 mM). Flow: 0.6 mL/min. Gradient: 90 %B to 60 %B in 10 minutes. Column temperature: 30 °C. Note: system required 0.5 % phosphoric acid wash to prevent tailing. Good peak shape can be maintained both by reducing the injection volume and by diluting samples in weaker solvents (such as acetonitrile). Diluted samples can be injected in larger volumes to increase signal, making this the preferred method for general use.

Detection

HILIC differs somewhat from traditional reversed-phase chromatography in terms of the detectors used. Mass spectrometry is the ideal detector for HILIC, while UV-Vis tends to be less applicable than in reversed-phase mode; finally, universal detectors such as the refractive index detector (RI) and evaporative light scattering detector (ELSD) are more common in polar analyses.

MS detection:

Mass spectrometry is an excellent detection method for polar analytes, which have high ionization efficiency and excellent sensitivity. Further, HILIC operates at high concentrations of organic and handles volatile buffers well, which makes electrospray ionization (ESI) highly efficient.

UV-Vis detection:

Many polar compounds do not contain chromophores: sugars, amino acids, and inorganic ions. This is because many UV-active groups (phenyl rings, esters, amides, C=C bonds) are rather nonpolar, while polar groups (-OH, -NH_2, C-O-C) have absorbance that overlaps with common buffers and solvents. Nonetheless, UV remains a cheap and sensitive option for compounds that contain a polar group with a strong UV response, such as organic acids.

Evaporative light scattering detection:

Evaporative light scattering detectors give excellent sensitivity and response for nonvolatile compounds such as sugars, metals, and large molecules. ELSD requires volatile buffers as with MS, but tolerates higher buffer concentrations, as well as the challenging conditions of sugar analysis (high pH or high temperature).

Volatile compounds, such as small organic acids or amines, are challenging to analyze by ELSD, and may require an alternative detector.

Refractive index detection:

Refractive index (RI) detectors can also be used to detect virtually any compound, but are less sensitive, and cannot handle gradient elution.

RI tolerates high buffer concentrations and can detect volatile compounds that would normally be lost on an ELSD. This makes them the detector of choice for mixtures containing volatile compounds, such as alcohols or small organic acids.

Other detection techniques:

Polar analytes are also measured with other detection methods, not currently covered by Agilent. These include:

- Conductivity detectors for inorganic ions
- Pulsed amperometric detectors (PAD) for sugars and amines
- Charged aerosol detector (CAD) for universal detection, which is similar to ELSD

Table 3. Summary of detection techniques.

Detector	Benefits	Common analytes	Limitations
UV-Vis	Common and inexpensive Sensitive Low dispersion	Analytes must have chromophore: aromatic ring, carboxylic acid, ester, and so on Nucleosides, nucleotides, organic acids	High noise with formate/acetate buffer at low wavelengths (about 210 nm) Limited sensitivity
LC/MS – Positive mode	Highest sensitivity	Amines and organic acids	Must use compatible buffer and low concentrations (about 10 mmol/L)
LC/MS – Negative mode	High sensitivity Alternative range of detection	Organic acids, phosphate compounds	Must use compatible buffer and low concentrations (about 10 mmol/L)
Refractive index	'Universal' detection Low cost Compatible with high buffer concentrations	Compounds and mixtures lacking chromophores Sugars, inorganic ions, amino acids	Low sensitivity Requires isocratic conditions Long start-up time
Evaporative light scattering	'Universal' detection Sensitive (similar to UV-Vis)	Compounds and mixtures lacking chromophores Sugars, inorganic ions, amino acids	Cannot detect volatile compounds (alcohols, organic acids) Must use volatile buffer Older models often have nonlinear calibration curves

Sticky samples, deactivation, and inert hardware

All grades and types of steel have a layer of metal oxides on the surface that prevents corrosion. These metal oxides also contain sites that bind to certain classes of sticky polar molecules.

Most active molecules:

- Phosphorylated metabolites and phosphate esters
- Organophosphates and phosphonic acids
- Di- and tri-carboxylic acids and chelating agents

Commonly seen in:

- Pesticide analysis (glyphosate, AMPA, glufosinate)
- Fermentation (citric acid cycle, organic acid monitoring)
- Metabolomics (nucleotides, sugar phosphates, citric acid cycle)
- Inorganic analysis (Fe monitoring, EDTA analysis)

When these compounds interact with the steel, they will often tail at high concentrations and disappear completely at low concentrations. Historically, analysis methods usually rely on derivatization, extreme pH, ion pairing, or competitive chelating agents to measure these classes of compounds.

A simple alternative to this approach is to deactivate the metal sites on the steel surface, which can be done using a mild phosphoric acid wash (0.5 % phosphoric acid in 90:10 acetonitrile:water). The phosphoric acid strongly bonds to the active sites on the system, enabling satisfactory analysis of sticky compounds. Generally, the pump head handling organic solvent (pump B) requires deactivation more frequently than the aqueous pump (pump A).

Sensitivity can be further enhanced by switching to 90:10 acetonitrile:water in the B phase bottle, and replacing glass bottles and vials with plastic, such as HDPE.

Finally, replacing steel components with PEEK or PEEK-lined stainless steel has a further benefit to the chromatography. PEEK-lined columns and capillaries are available for Agilent systems and columns.

Option	Details
1. Use 10 % aqueous phase in acetonitrile	100 % acetonitrile can interact with steel and introduce impurities. Adding 10 % aqueous eliminates this.
2. Wash in 0.5 % phosphoric acid	Washing in a mild phosphoric acid solution temporarily deactivates the active sites on steel.
4. Switch to plastic solvent bottles and vials	Eliminates signal suppression from Na, K, Ca, BO_3, and SiO_4 leaching from laboratory glass
3. Switch to PEEK-lined hardware	Replacing steel with PEEK-lined hardware reduces the number of binding sites (but rarely eliminates them all, so washing may still be needed on occasion).

Step-by-step system wash procedure

Hands-on time: 30 minutes

Total wait time: Three hours plus overnight (approximately 15 hours)

1. Set the MS so that the source can be handled safely. This is normally the same setting that would be used for source cleaning (see notes below).
2. Change the flow rate to 0 mL/min, and switch the solvent to pure water.
3. Set the system to Purge On, flowing directly to waste, or remove the inlet capillary from the HPLC column and place it in an appropriate waste container (see notes below).
4. Purge the HPLC pump with water at 5 mL/min for five minutes.
5. Set the system to Purge Off or stop the flow and reconnect the HILIC column
6. Set the flow of water to 0.5 mL/min for 4.6 and 3.0 mm diameter columns, or to 0.25 mL/min for 2.1 mm diameter columns. Run for 30 minutes through the system and column.
7. Change the flow rate to 0 mL/min and switch the solvent to 0.5 % phosphoric acid (in 9:1 acetonitrile:water)
8. If using an MS or other detector with a nebulizer, detach and remove the spray needle and place it spray-end down into an appropriate waste container, then reconnect the inlet capillary (see notes below).
9. Set the system to Purge On, flowing directly to waste, or remove the inlet capillary from the HPLC column and place it in an appropriate waste container.
10. Purge the HPLC pump with 0.5 % phosphoric acid at 5 mL/min for five minutes.
11. Set the system to Purge Off or stop the flow and reconnect the HILIC column.
12. Set the flow of 0.5 % phosphoric acid at 0.1 mL/min, and run overnight (12 hours minimum).
13. Change the flow rate to 0 mL/min, and switch the solvent to pure water.
14. Set the system to Purge On, flowing directly to waste, or remove the inlet capillary from the HPLC column, and place it in an appropriate waste container.
15. Purge with water at 5 mL/min for five minutes.
16. Set the system to Purge Off or stop the flow, and reconnect the HILIC column.
17. Set the flow of water to 0.5 mL/min for 4.6 and 3.0 mm diameter columns, or 0.25 mL/min for 2.1 mm diameter columns. Run for one hour through the system and column.
18. Change the flow rate to 0 mL/min, and switch the solvent to the desired mobile phase.
19. Set the system to Purge On, flowing directly to waste, or remove the inlet capillary from the HPLC column and place it in an appropriate waste container.
20. Purge with mobile phase at 5 mL/min for five minutes.
21. Set the system to Purge Off or stop the flow and reconnect the HILIC column.
22. Set the flow of mobile phase to 0.5 mL/min for 4.6 and 3.0 mm diameter columns, or to 0.25 mL/min for 2.1 mm diameter columns. Run for one hour through the system and column.
23. Reconnect the nebulizer to the MS, and proceed with the analysis.

Notes:

- Contact technical support for the MS manufacturer if there is any doubt on how to appropriately handle the source.
- An appropriate waste container should be clean, empty, compatible with the solvent, and large enough to hold the waste solvent without spillage. A large glass beaker or solvent bottle is generally recommended.
- The ESI needle capillaries, and any other components should not become immersed in solvent or waste. Containers should be emptied regularly.
- If the nebulizer needle cannot be removed easily, the same steps can be followed with the capillary end that connects to the MS system. However, some interactions may occur within the needle.
- Passivate as much in the flowpath as possible: HPLC system, capillaries, column, and detector.
- Use appropriate safety measures when handling all solvents and HPLC components.
- DO NOT run phosphate into the MS.

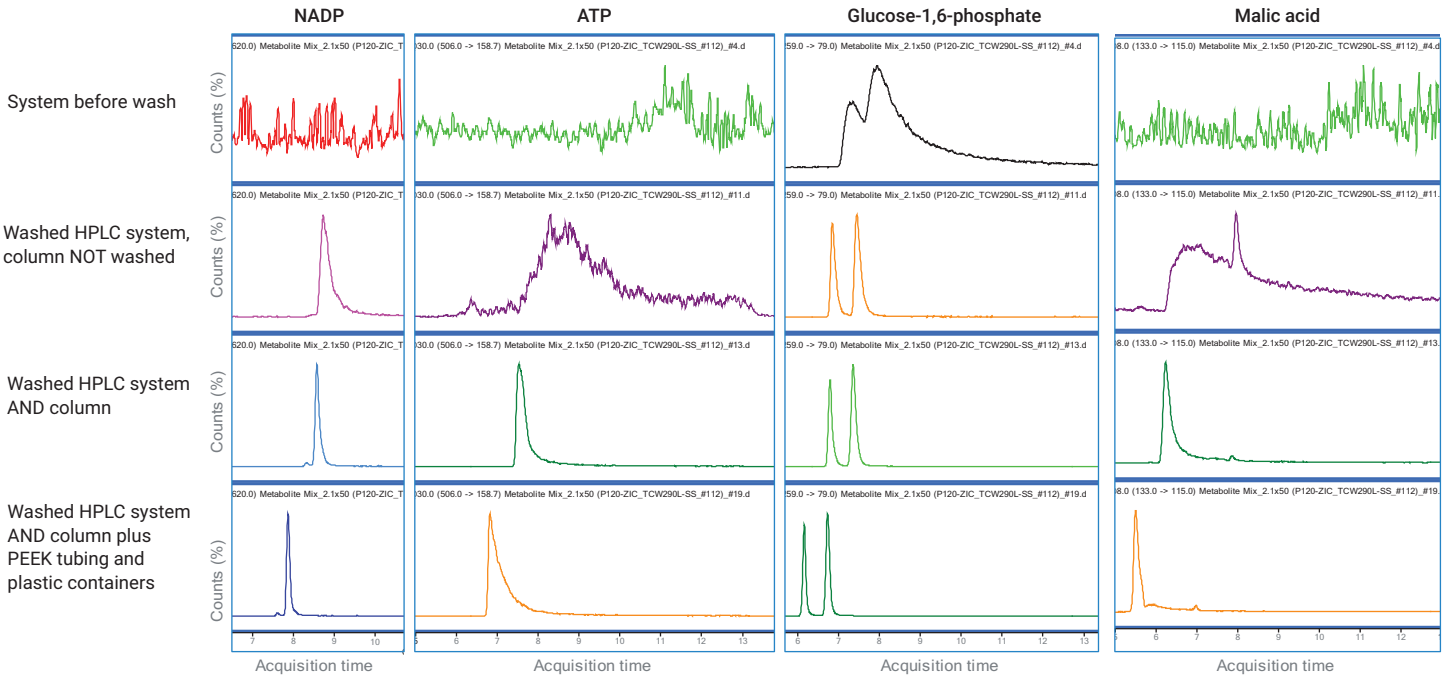

Figure 9. Interactions of phosphorylated metabolites with steel: before and after wash. Column: InfinityLab Poroshell 120 HILIC-Z (PEEK-lined stainless steel), 2.1 × 100 mm, 2.7 µm. Mobile phase A: 10 mM ammonium formate in water, pH 6.8. Mobile phase B: acetonitrile + 10 mM ammonium formate, pH 6.8. Gradient: 95 to 30 %B in 10 minutes. Flow: 0.25 mL/min. Injection: 0.2 µL (5 ng each on column). Detection: MS, ESI source, negative mode.

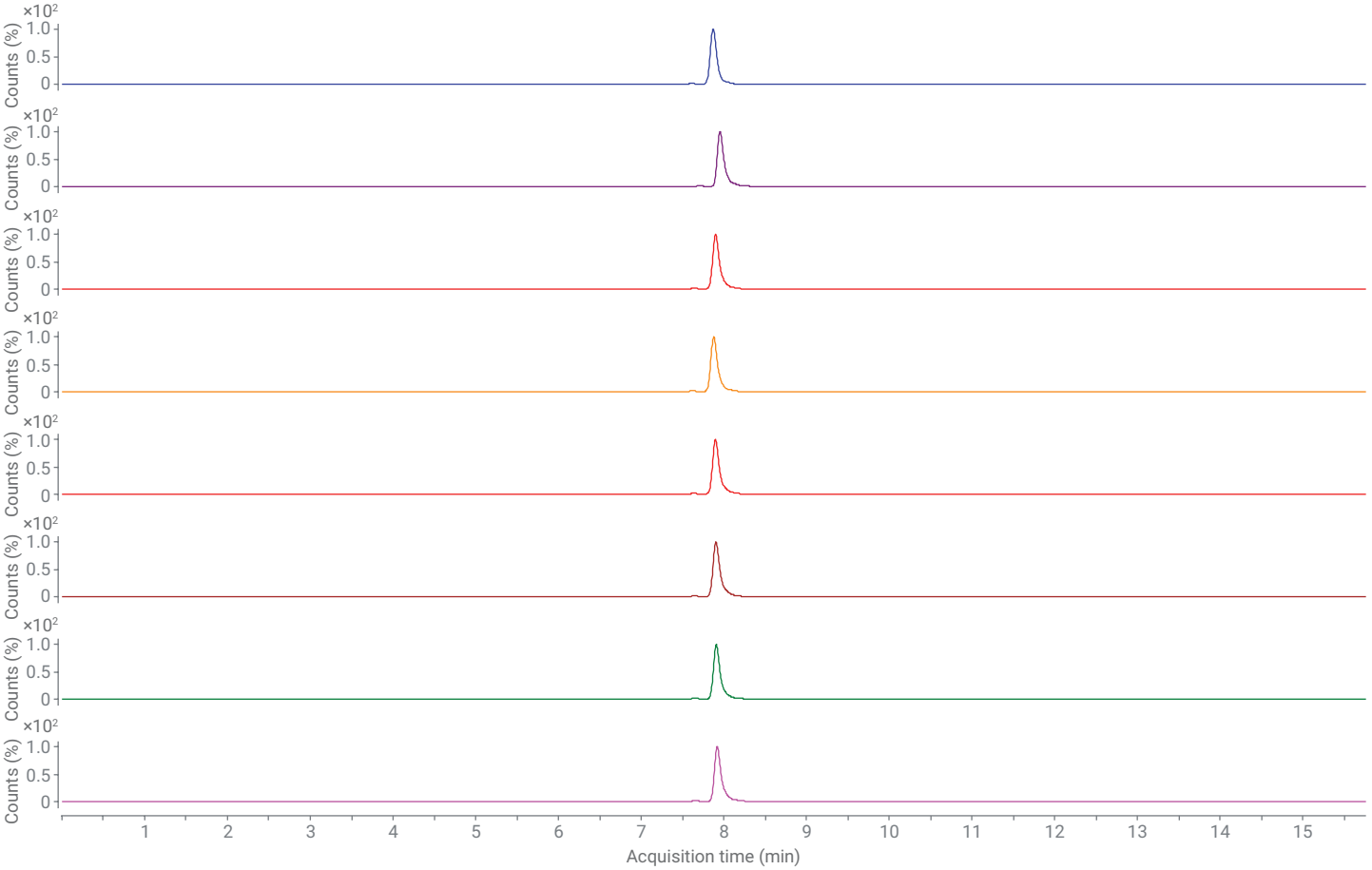

Figure 10. Reproducibility of NADP postwashing shown by consecutive injections after eight hours postpassivation.

Troubleshooting

Certain issues, such as reproducibility and equilibration, were sometimes thought to be inherent weaknesses of HILIC. In fact, these can be eliminated with proper troubleshooting.

Slow re-equilibration – the most common issue

Root cause	Lack of water in mobile phase
Resolution	Review section "Getting Started with HILIC". The mobile phase *must* have enough water to refresh surface layer every run. To ensure rapid re-equilibration, the column should be run with at least 20 % water, and 50 % is recommended.

Reproducibility – the second-most common issue

Root cause	Not re-equilibrated before next run Sample solvent is too strong, affecting column equilibration Interaction with active sites on the stainless steel in the system
Resolution	Column is not fully re-equilibrated. Review section "Getting Started with HILIC". Mobile phase should have enough water to quickly refresh surface layer every run, otherwise re-equilibration time must be extended. Sample solvent is impacting retention. Review section "Getting Started with HILIC". Increase organic content of sample solvents and/or reduce injection volumes Target analyte is adsorbing to the steel in the system. Review section "Sticky Samples, Deactivation, and Inert Hardware". If the sample falls into one of the categories of 'sticky' compounds, consider a phosphoric acid wash and inert hardware.

Sample solubility – sample-specific issue

Root cause	High organic solvents are ideal sample solvents, but can be poor solvents for salts and other polar analytes.
Resolution	Review section "Getting Started with HILIC". Dilute samples in acetonitrile as much as solubility allows, then reduce injection volumes for acceptable peak shape and reproducibility.

Peak shape – sample-specific issue

Root cause	Peaks can split or tail if large volumes of strong solvent are injected into the system. Samples may tail when they stick to active sites on the steel in the system. Some HILIC phases, such as silica, may have secondary ion exchange interactions, which cause excessive retention and tailing of anions or cations.
Resolution	Dilute sample in weaker solvent (acetonitrile). Review section "Getting Started with HILIC". If the sample falls into one of the categories of 'sticky' compounds, Review section "'Sticky' Samples, Deactivation, and Inert Hardware". Consider a system wash and inert hardware. Switch bonded phases. Review section "Getting Started with HILIC". Secondary retention is common in older HILIC phases. Increase buffer concentration. Review section "Getting Started with HILIC". Adding buffer improves peak shape, but can impact sensitivity in MS and baseline stability in ELSD and UV.

Buffer solubility

Root cause	High organic solvents necessary to retain less polar analytes in HILIC mode, but many buffers are poorly soluble.
Resolution	A small amount of water increases buffer solubility in acetonitrile significantly. Review section "Getting Started with HILIC". Add 10 % water to acetonitrile to improve solubility in general, and always test the solubility of mixtures containing high buffer concentration with high organic concentration.

Conclusions

HILIC is a robust and reliable choice for analysis of even the most polar compounds. HILIC is easily implemented in any lab currently using reversed-phase chromatography, but has a few distinctive differences that should be well understood before developing or adopting a HILIC method.

Key takeaways include:

- Solvent strength is reversed in HILIC, both for eluents and sample solvents. This can be a source of initial confusion even for experienced chromatographers.
- Mixtures with a high percentage of organic can be poor solvents for polar compounds. Check the solubility of samples and buffer salts in high acetonitrile solvents to prevent sample loss or clogs.
- Fast equilibration and excellent reproducibility are possible with proper method conditions. Users must understand the connection between the water layer on the particle and the speed of equilibration.
- Polar compounds are much more likely to interact with stainless steel, making issues with 'sticking' much more common in HILIC than traditional reversed-phase analysis. Common symptoms are severe tailing and signal loss at low concentrations. A mild phosphoric acid wash deactivates many of the active sites on steel, while switching to inert hardware eliminates them altogether.

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